Exhibit A

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RNA-triggered gene silencing

Double-stranded RNA (dsRNA) has recently been shown to trigger sequence-specific gene silencing in a wide variety of organisms, including nematodes, plants, trypanosomes, fruit flies and planaria; meanwhile an as yet uncharacterized RNA trigger has been shown to induce DNA methylation in several different plant systems. In addition to providing a surprisingly effective set of tools to interfere selectively with gene function, these observations are spurring new inquiries to understand RNA-triggered genetic-control mechanisms and their biological roles.

Andrew Fire
fire@
mail1.ciwemb.edu

Carnegie Institution of Washington, 115 West University Parkway, Baltimore, MD 21210, USA.

As gene-transfer technologies have become commonplace, an increasing number of organisms have been shown to exhibit potent and unexpected responses to foreign nucleic acids. The ability of some transgenes to silence the expression of homologous (chromosomal) loci was first observed in plants[1] and has subsequently been seen in nematode[2], fungal[3], insect[4] and protozoan[5] systems. Homology-dependent *trans*-silencing effects (see Box 1 for glossary) have been divided into two categories based on the nature of the effect on the target. In the first category, transcription of the target locus is unaffected, whereas the half-life of target RNAs is decreased dramatically[6–9]. Such processes have been called 'PTGS' (post-transcriptional gene silencing). A second category of homology-triggered processes exert their primary effect on the chromatin template[10], and have been termed 'TGS' (transcriptional gene silencing). A striking feature of PTGS, and of a subset of TGS phenomena, has been the existence of RNA trigger molecules responsible for the long-range effect of the transgene locus on the endogenous gene. This article will attempt to describe some emerging views, first of RNA-triggered PTGS and then of RNA-triggered TGS, while highlighting the many mechanistic questions that remain to be resolved.

BOX 1. A lexicon of gene-silencing effects

Antisense interference
Blocking the activity of genes by artificially providing complementary single-strand antisense nucleic acid corresponding to the target gene.

Cosuppression
The ability of some transgenes to silence themselves and homologous chromosomal loci simultaneously.

dsRNA-triggered interference
Blocking the activity of genes by artificially providing sense and antisense RNA corresponding to a target gene.

Homology-dependent trans silencing
The ability of an RNA or DNA trigger to silence a corresponding chromosomal locus in *trans* (i.e. without any genetic linkage to the target locus).

Post-transcriptional gene silencing (PTGS)
The ability of some viruses, transgenes or RNAs to trigger the post-transcriptional degradation of homologous cellular RNAs.

Quelling
A cosuppression phenomenon in *Neurospora crassa*.

Repeat-induced gene silencing (RIGS)
(Not discussed in this paper but included in this box for clarity.) In general, RIGS refers to a localized (*cis*-acting) effect, in which regions of tandemly repeated sequence are silenced, frequently without silencing homologous genes at other sites in the genome.

RNAi
The ability to block activity of a cellular gene by injection of homologous RNA (generally used in *Caenorhabditis elegans*).

PTGS as part of a primitive immune system

In the real world, the genome of an organism must survive in a hostile environment with dangerous opportunities for unwanted gene expression and with parasites (transposons and viruses) whose interests are distinct from those of the host. This antagonism creates a need for global mechanisms that limit aberrant or unwanted gene expression. Mechanisms that are used for global genome surveillance are likely to encompass such diverse phenomena as nonsense-mediated mRNA decay[11], intron requirements for mRNA biogenesis[12], preferential methylation of transposon sequences[13] and repeat-dependent silencing[14], as well as the RNA-triggered silencing processes described in this review. In each case, a nucleic acid conformation that is not associated with normal gene expression is used by the organism as a means to recognize potentially problematic situations; in each case, one result is to block the production or expression of potentially harmful RNAs.

The best-understood biological role for an RNA-triggered silencing process comes from studies in plant systems, where a series of observations now strongly implicate PTGS as an antiviral mechanism. (1) Viral RNAs can be targets for PTGS (Ref. 1). (2) Intercellular spreading of the PTGS effect allows the plant to respond in a systemic manner after a localized viral challenge (e.g. Refs 15–17). Intracellular spreading of PTGS is thought to involve the direct dissemination of interfering RNA in a form that is reserved for genetic interference. Note that a similar spreading of the PTGS effect is also seen in *Caenorhabditis elegans* after extracellular delivery of dsRNA (Refs 18–20). (3) Several different plant viruses produce proteins that can generally interfere with PTGS (Refs 21–24). (4) Plant strains lacking PTGS are apparently healthy under laboratory conditions[21,22,25]. These strains were made using transgenic technology to produce viral anti-PTGS factors[21,22] or by direct genetic selection for lack of a PTGS response[25]. (5) Some viral infections are more harmful to plants that lack an effective PTGS response[21,22].

Although these experiments clearly implicate PTGS in an antiviral response, it is quite possible that roles will be found in control of other genomic parasites, as well as in more general aspects of cellular physiology.

Self versus non-self RNA: the nature of the PTGS trigger

Cells survive by unimpeded expression of their own genes. This implies that an RNA trigger for gene silencing must be recognized as distinct from normal cellular RNA. One potential character that might implicate RNA as foreign would be a double-stranded structure[26]; double-stranded RNA (dsRNA) is not a requisite product of normal gene expression but is produced (at least transiently) by many viruses. Strikingly, exogenous dsRNAs can act as potent triggers of PTGS in nematode[9,18], trypanosome[8], insect[27,28] and planarian[29] systems. Measurements of dsRNA potency in *C. elegans* and *Drosophila* indicate that a few molecules per cell suffice to trigger a vehement PTGS response[9,27]. Two different methods that should produce dsRNA in plants also provide reproducible triggering of PTGS: (a) the simultaneous expression of sense and antisense sequences corresponding to the desired target gene[30] (or of an RNA hairpin[30,31]) and (b) the simultaneous expression of a viral RNA replicase with a specific single-stranded RNA (ssRNA) that has been engineered to contain viral replication signals[32]. (In the latter case, note that dsRNA has been suggested but not proven as the key feature in triggering PTGS.) Because these experiments involve the deliberate introduction of aberrant RNA, they leave open several questions regarding the general nature of the PTGS trigger: (1) when and how dsRNA forms in the cell; (2) whether the formation of dsRNA is always sufficient to trigger a PTGS response; and (3) whether all PTGS involves a dsRNA trigger.

One intriguing aspect of PTGS has been the ability of transgenes that are designed to produce only sense or only antisense RNA to act as triggers[1,2,7]. Low levels of dsRNA might be produced in such cases, through the spurious transcription of both strands of a transgene, or through the transcription of inverted repeats that sometimes form when transgenes integrate[33]. As an alternative, it now seems possible that cellular RNA-dependent RNA polymerase (RdRP) could be involved in producing RNAs that can trigger PTGS. Although RdRP activity had been observed in crude extracts from several different cell types, details of the activity became clearer after an enzyme was purified and cloned from Tomato[34,35]. Genetic studies in *Neurospora* support the involvement of an RdRP in some aspect of PTGS. Screens for the loss of PTGS response to certain 'sense' transgenes led to the isolation of several mutations, one of which has been shown to result from the disruption of an RdRP-related gene[36]. Although the *Neurospora* gene product has not yet been shown to possess RdRP activity, the genetic and biochemical analysis provides an exciting link between observations in the different systems. The reaction catalyzed by the Tomato RdRP *in vitro* is a relatively non-specific conversion of

ssRNA to dsRNA. The *in vivo* specificity is likely to be highly regulated, because general copying of all cellular RNAs would wreak havoc. Analyses of the pattern of 'sense' and 'antisense' transgenes that are capable or incapable or inducing PTGS have led to several models for *in vivo* specificity for RdRP (Ref. 37). Characteristics of sense transgenes that might trigger PTGS have been proposed to include overproduction of normal RNAs, specific intramolecular secondary structure, truncations in RNA, and ineffective transcription or translation, all of which have been cited. An enticing model is that these conditions all lead, by some means (RdRP or symmetric transcription), to the production of extended regions of dsRNA.

Clearly, there needs to be tight control of any mechanism producing a trigger for PTGS. The potent response to dsRNA could pose problems for the organism, in that the accidental production of a few antisense transcripts for an important gene could provoke an unintended PTGS response. At an evolutionary level, this would be partially controlled by selection against cryptic opposite-strand promoters. Even if some accidents occurred, the combined synthesis of sense and antisense RNA in a single cell does not guarantee the formation of an interfering dsRNA. Formation of dsRNA depends on the ability of the two strands to find each other and hybridize within the dense environment of RNA-binding proteins that are present in the cell, and on the ability of the resulting duplex to resist enzymes that unwind, covalently modify, or degrade duplex RNA. Additionally, some dsRNAs might be less effective in triggering PTGS; in particular, cells might have a strong interest in triggering PTGS from foreign or aberrant dsRNA (which would signal infection) in preference to duplexes containing their own mRNA. Consistent with this hypothesis are observations in *C. elegans* (Ref. 18) and *Trypanosoma brucei* (Ref. 8), which demonstrate that triggering of PTGS by direct introduction of foreign RNA requires that both the sense and the antisense strands are provided exogenously, even if a cell already has a substantial pool of naturally synthesized sense mRNA. This is also true in plants[30], although high-level expression of sense and antisense RNAs from distinct chromosomal sites can be sufficient, in this case, to produce a PTGS response.

Can any RNA be a target of PTGS?

In each system examined, numerous mRNAs can be targets of dsRNA-triggered PTGS (Refs 8, 18–20, 27–31). Characterized targets include newly synthesized (nuclear) RNA (Ref. 9) and pre-existing cytoplasmic RNA (Ref. 8). Targeting of some nuclear RNAs argues against the direct involvement of the translational machinery, while the ability to target infecting viral RNA (Ref. 30) argues against any linkage to DNA transcription within the cell. Although sensitivity to dsRNA-triggered PTGS appears to be the rule rather than an exception, there might be some target RNAs that partially or fully resist PTGS (Ref. 20). Similarly, some tissues might be partially resistant to the effect (including some parts of the *C. elegans* nervous system; J. Fleenor and A. Fire, unpublished). Further investigation of resistant genes and cells could illuminate the study of (a) tissue- and sequence-specific aspects of the PTGS machinery, and (b) the distribution and properties of enzymes that might degrade or unwind the dsRNA trigger.

Because PTGS acts by decreasing the half-life of RNA, the natural stability of an RNA will have a quantitative influence upon its suitability as a PTGS target: naturally stable RNAs are likely to be more dramatically affected, whereas RNAs that are rapidly synthesized and degraded might be less affected. Homeostatic regulation mechanisms might also influence the final outcome of PTGS, in that a decrease in final product could activate metabolic compensation mechanisms that would partially restore expression level.

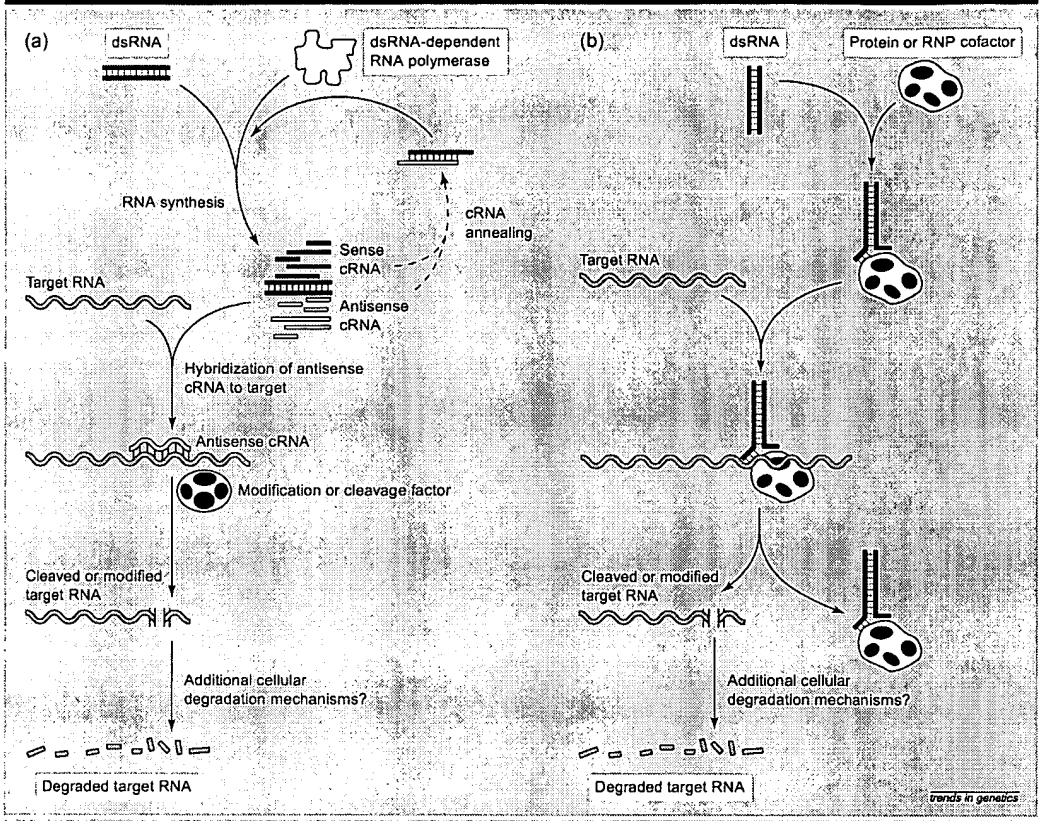
Still a mystery: mechanisms for PTGS

The ability of a few molecules of dsRNA to eliminate a much larger pool of endogenous mRNA (Refs 18, 27) suggests a catalytic or amplification component to the interference mechanism. Some of the plant literature (e.g. Refs 15, 30, 37) has favored an RNA-based copying system that is proposed to produce copious amounts of antisense RNA (while perhaps also producing additional sense and dsRNA). Direct evidence for such copy RNA (cRNA) has not been reported, although the role of an RdRP-related gene product in 'sense'-induced PTGS in *Neurospora* is certainly intriguing in this regard. Purified RdRP from Tomato is not capable of copying a dsRNA template in isolation[34]; nonetheless, it is reasonable to assume that additional enzymes could help to unwind the dsRNA product. As noted above, RdRP might alternatively (or also) contribute to PTGS at an earlier step (generating dsRNA from aberrant or unwanted ssRNA). In the absence of evidence for abundant cRNA copies of triggering RNA, an alternative to amplification is also considered. This model[9] involves the formation of a catalytic multi-round RNA-degradation machine from each dsRNA molecule. These two models (not mutually exclusive) are contrasted in Fig. 1.

To generate specificity, the initial interaction with target RNA is likely to involve hybridization, at least in a limited region. In the cRNA model of Fig. 1a, this is achieved by producing many copies of antisense RNA, which then find target RNA by hybridization. If no direct amplification is involved, then the original dsRNA must participate directly in recognition, implying a partial unwinding of the incoming RNA duplex.

After an RNA has been recognized as a PTGS target, the next challenge is to explain its decay. Recent studies of PTGS in tobacco have refined the proposal that an endonucleolytic cleavage is a key step in degradation of the target mRNA (Ref. 38). There are several ways that the initial (hybridization-based) interaction could lead to such an endonucleolytic cleavage. The least-complex model would be direct cleavage by a dsRNA-specific nuclease. Alternatively, the initial duplex might serve as a recognition site for an enzyme that then modifies the target RNA. The best candidate for such a modification would be the de-amination of adenosines to inosines, a reaction known to be catalyzed in dsRNA regions by enzymes of the ADAR family[39]. Finally, it is possible that the effect of the initial interaction with the target RNA is not a covalent change, but a positional change (sequestering the target RNA to an inhospitable region of the cell) or alteration to the spectrum of bound proteins. Whatever the initial act that modifies the target RNA, its eventual (complete) degradation is likely to require additional mechanisms. This might involve a combination of PTGS-specific degradation pathways and normal RNA decay mechanisms.

FIGURE 1. Two models for post-transcriptional gene silencing



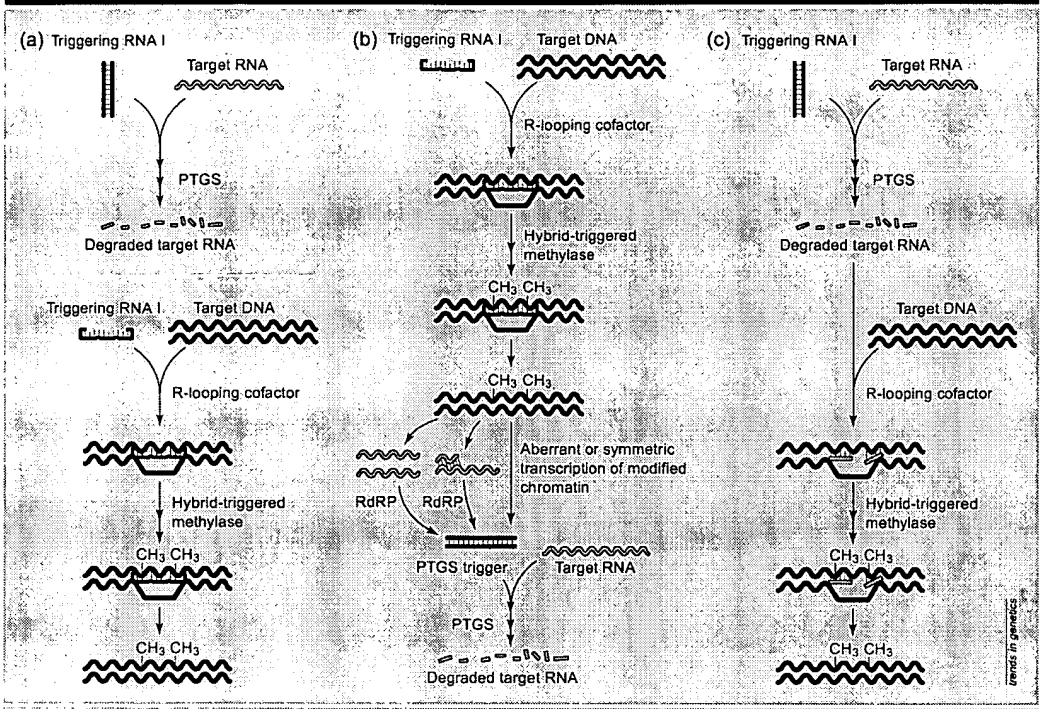
(a) A model postulating amplification of the double-stranded (dsRNA) trigger through synthesis of copy RNA (cRNA) by an RNA-dependent RNA polymerase (based on models in the plant literature; see Refs 30, 37). (b) A model that does not postulate copying once dsRNA has formed but, rather, the activity of a multi-round enzyme that can trigger the decay of many molecules of target RNA (based on Ref. 9). Unwinding of the interfering dsRNA is shown (arbitrarily) at an end of the duplex: in principal this could occur anywhere along the length of the molecule.

Is RNA-triggered chromosome modification related to PTGS?

In several plant systems it has been shown that RNA-triggered genetic silencing is accompanied by cytosine methylation (at the DNA level!) for portions of the target gene[16,40-43]. The structural identity of the endogenous or exogenous RNA that is directly responsible for triggering the modification of DNA is not known in any instance; this will be a major question of interest in the next few years. Although viral replication complexes that include dsRNA or inverted repeat transgenes are present in a number of these situations[16,40,42,43], it is conceivable that the process is completely distinct from PTGS and results from an independent interaction between the interfering RNA and the template DNA (the 'two independent process' model shown in Fig. 2a). A second model proposes one type of causal relationship, with RNA-triggered methylation of DNA causing aberrant transcription, and with the resulting transcripts inducing PTGS (Fig. 2b). Conversely, the methylation of target DNA sequences might happen as a consequence of PTGS: this model proposes that degradation products of the target mRNA (i.e. pieces of the target RNA produced by PTGS) would interact with the gene (Fig. 2c). A related model would be one in which large amounts of degraded RNA produced by PTGS might hybridize to the corresponding DNA template, causing a delay in replication that might be sufficient to induce a heritable change in the activity state of the template. If there are common components in PTGS and RNA-triggered chromosome modification, it seems likely that they will be identified by genetic analysis. In particular, it will be interesting to know the molecular identity and role of two genes in *Arabidopsis* that have effects on PTGS and on transgene-triggered DNA methylation[25].

For any model to account for sequence specificity in RNA-triggered DNA methylation, some type of RNA–DNA hybridization would be a likely component. From an experimental perspective, an observer might expect to find (1) heteroduplexes (perhaps transient) between DNA from the affected region and aberrant RNAs and (2) an enzyme that could recognize these heteroduplexes as substrates for the methylation of cytosines (or other chromosome modification, such as histone deacetylation or binding of repressive protein complexes). In cases where regions of a target gene become methylated, what is this effect on gene expression? This question adds a level of complexity, because effects on expression will depend on the exact positioning of methylated cytosines, relative to control

FIGURE 2. Possible links between PTGS and RNA-triggered chromosome modification



(a) A model postulating separate modes of action for post-transcriptional gene silencing (PTGS) and RNA-triggered chromosomal modification. An RNA of unknown structure is drawn as triggering the latter response. (b) A model in which RNA triggers DNA methylation, leading to aberrant transcription and resultant triggering of PTGS. (c) A model in which target RNA decay products from PTGS interact with the DNA template to produce a heritable change. Primary modifications to the chromosome in the diagram are arbitrarily shown as involving methylation of DNA. Alternatively, the initial modifications could involve local changes in histone modification or specific chromatin factors.

regions. The existing data from plants indicate that the heaviest RNA-triggered DNA methylation is restricted to sequences that are present in RNA (Refs 40–43). In situations where the homology between trigger and target is only within coding sequences, the methylation of the target might leave promoters and enhancers unaffected, with minimal consequent effects on expression. Under these circumstances, a strong effect would require either: (1) the presence of promoter or enhancer elements in the coding region; (2) a mechanism to nucleate a spreading of chromosomal modification from the coding region to nearby enhancers and promoters (limited spreading has been observed for viroid-induced DNA methylation in Tobacco[43]); or (3) the ability of methylation within coding sequences to impede transcriptional elongation (this has been shown for *Neurospora*)[44]. Strong effects on gene expression would be expected for triggering RNA populations that include promoter and/or enhancer sequences; this was recently demonstrated in transgenic Tobacco by Mette *et al.*[42], who showed that an active promoter could be *trans*-silenced by expression (from a second transgene) of aberrant RNAs covering the promoter. Whatever the mechanism and magnitude of the effect on gene expression, a general ability of aberrant RNAs to alter the original template could have utility in allowing a chromosome to respond to the consequences of its actions (in particular, the ongoing production of an aberrant RNA).

What is the evolutionary extent of RNA-triggered genetic silencing?

Given the diverse phylogenetic positions of plants, nematodes, fungi, flies, planaria and trypanosomes, it seems certain that the distribution of RNA-triggered genetic silencing in the biosphere is extensive. Examples are likely to accumulate as gene transfer (or RNA injection) is attempted in additional organisms. The initial observation will often be *trans*-silencing by foreign or engineered DNA. Frequently, the lack of a promoter in the incoming DNA (or failure to find RNA transcripts) is taken as evidence for lack of an RNA intermediate: this type of evidence should be interpreted cautiously, because transgenes are frequently transcribed at low levels on both strands and a few molecules of dsRNA would rarely be detected in these experiments. Some *trans*-acting genetic effects are definitively not triggered by RNA (e.g. transvection effects in *Drosophila* triggered by DNA–DNA pairing[45]). Nonetheless, it seems prudent to carefully consider a possible RNA trigger (particularly dsRNA) as each new homology-dependent *trans*-silencing phenomenon is characterized.

Real-world applications: what about us?

Procedures based on RNA-triggered silencing are now well-established tools for functional genomics of lower organisms (plants, invertebrates and fungi). Valuable information about gene function can be obtained, even in

cases where only a partial loss-of-function is generated. From a technical perspective, one could certainly hope that RNA-triggered silencing would exist in vertebrates: this would facilitate functional genomics and might allow medical applications involving targeted silencing of 'renegade' genes. Although this hope is not ruled out by any current data, the simple protocols used for invertebrate and plant systems are unlikely to be effective. Mammals have a vehement response to dsRNA, the best-characterized component of which is a protein kinase (PKR) that responds to dsRNA by phosphorylating (and inactivating) translation factor EIF2a (Ref. 46). As with nematode and plant systems, mammalian cells can respond to extracellular dsRNA and, thus, might have a specific transport mechanism to bring the dsRNA to the intracellular PKR enzyme. Controlled-delivery studies suggest that a single molecule of dsRNA within the cell can trigger an overall cellular response[47]. Any gene-specific dsRNA response in mammals would need to exist in cells or conditions where PKR is less effective, or would need to work in the shadow of the PKR-induced global response. Nonetheless, a recent report of co-suppression in mammalian cells[48], and the implication of RNA triggers with a potentially double-stranded character in a number of natural genetic interference processes (X-inactivation[49] and imprinting[50]) suggest the possibility that some components of RNA-triggered silencing machinery could be conserved from lower organisms.

Even if the underlying mechanisms are absent in mammals, it is possible that RNA-triggered silencing will have clinical applications. In particular, the ability to silence essential parasite genes (thereby limiting a parasitic infection) could be of great value. Of course, the dsRNA would have to be delivered so as to avoid harming the host. The PKR system (although non-essential for survival in mouse models[46]) is sufficiently ubiquitous that interfering with it might be counterproductive. An alternative would be to find chemical modifications to the dsRNA that would still enable it to function in gene-specific interference (e.g. in a parasite), while not inducing the PKR response in the host.

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